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# Immunohistochemical staining of CD44 core proteins in islet beta cells of formalin-fixed mouse pancreas V.2

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**We use this protocol and it's working**

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## Abstract

Paraffin sections (4mm thickness) of formalin-fixed mouse pancreases were treated with heat/citrate buffer for antigen retrieval. CD44 core proteins were detected immunohistochemically using rat anti-mouse CD44 mAb (IM7 mAb; BD Biosciences) or mouse anti-human CD44v3 mAb (CD44v3: R&D Systems), with horseradish peroxidase-conjugated rabbit anti-rat Ig (Dako) or rabbit anti-mouse Ig (Dako). Background staining was checked using the corresponding isotype control Ig instead of the primary antibody. 3-amino-9-ethylcarbazole (AEC) was used as the chromogen. For morphometry, stained sections were imaged using a light microscope with attached camera (Olympus BX41). Image J software with color deconvolution plugin was used for the quantitative analysis of the % of islet area stained.

## Guidelines

Rat anti-mouse CD44 (IM7) mAb recognises all CD44 isoforms (1-4) and mouse anti-human CD44v3 mAb recognises a CD44 isoform that carries HS side-chains (1-3). In mouse pancreas we have demonstrated that these two mAbs have a similar pattern of intra-islet staining.

### References:

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## Troubleshooting

### Before start

#### Materials:

1. Prepare graded alcohols and xylene for deparaffinizing tissue sections: 2 x xylene (250 ml/slide container), 2 x absolute ethanol (250 ml/slide container), 1 × 90% ethanol (250 ml), 1 × 70% ethanol (250 ml).
2. Prepare acetate buffer components:
  - (i) 0.1N acetic acid: 290 µl glacial acetic acid in 50 ml deionized water
  - (ii) 0.1M sodium acetate: 410 mg anhydrous CH<sub>3</sub>COONa in 50 ml deionized water.Prepare 0.1M acetate buffer (pH 5.2) by mixing 10.5 ml 0.1N acetic acid and 39.5 ml 0.1M sodium acetate.
3. Prepare stock solution of 3-amino-9-ethylcarbazole (AEC; chromogen, 8 mg/ml: 40 mg AEC in 5 ml N-N-dimethyl formamide; protect from light and refrigerate at 4°C.
4. Prepare M.O.M. diluent: 200 µl M.O.M. protein concentrate stock solution (M.O.M immunodetection kit) in 2.5 ml phosphate-buffered saline (PBS) for use either as a blocking step to minimize non-specific Ig binding or for diluting antibodies.
5. Mabs and pAbs:
  - rat anti-mouse CD44 (IM7) mAb, BD Biosciences #553130 or
  - mouse anti-human CD44v3 mAb, R&D systems BBA11
  - horseradish peroxidase (HRP)-conjugated rabbit anti-rat Ig, Dako #PO450 (alternative HRP-rabbit anti-rat IgG, Sigma #A5795)
  - or HRP-rabbit anti-mouse IgG, Invitrogen #31450 (for use with CD44v3 mAb as primary ab)
  - Purified NA/LE Rat IgG<sub>2bK</sub>, BD Biosciences #555845 or
  - Purified Mouse IgG<sub>2bK</sub>, BD Biosciences#557351
6. Other reagents:
  - Hydrogen peroxide (30% w/w), Chem-Supply Pty Ltd (Australia) #HA154-500M,
  - Methanol, Merck # CAS-No. 67-56-1
  - 3Amino-9-ethylcarbazole (AEC), Sigma-Aldrich #A5754
  - Animal free blocker, Vector Labs #SP-5030
  - Stock protein concentrate, M.O.M immunodetection kit, Vector Labs # PK-2200
  - N-N-dimethyl formamide, Sigma #D158550
  - Glycergel mounting medium, Dako #C0563

- 1 See Guidelines before starting
- 2 Deparaffinize slides in each xylene for 1 min. rehydrate slides in graded alcohols beginning in absolute ethanol (10 dips)/ container of absolute ethanol), followed by 90% ethanol (10 dips) and 70% ethanol (10 dips).Wash well in running tap water for 5 min.
- 3 Wipe around sections with a tissue, encircle the sections using a diamond pencil and place in a slide container of tap water (250 ml).
- 4 Block endogenous peroxidase activity by incubating sections in 3% hydrogen peroxide in methanol (25 ml 30% H<sub>2</sub>O<sub>2</sub> + 225 ml methanol).
- 5 Wash 2 × 2 min in 250 ml phosphate-buffered saline (PBS) followed by wash in running tap water for 5 min.
- 6 Prepare citrate buffer, pH 6 for antigen retrieval. Dissolve 1.05 g Citric acid in 500 ml deionized water and pH using 2 - 10 M NaOH.
- 7 Transfer slides to 250ml citrate buffer and heat in microwave (1600 watt) for 2 min on High power followed by 2 × 6 min on Low power.Allow the slides to cool on the bench for 30 min. Wash slides in 250 ml PBS, 3 × 10 min.
- 8 Wipe around sections using tissue. To block non-specific binding of Ig, apply animal free block (diluted to 20% v/v with deionized water) to tissue sections and incubate for 10 min at room temperature.
- 9 Tip off excess block in Step 8, wipe around sections using tissue and incubate with 40 µg/ml anti-CD44 (IM7) mAb (or 40 µg/ml rat IgG<sub>2bK</sub> as isotype control; diluted in M.O.M. diluent), 125-150 µl/section at room temperature for 1 hour. Alternatively use 10 µg/ml mouse anti-human CD44v3 mAb (or 10 µg/ml mouse IgG<sub>2bK</sub> as isotype control Ig.  
Note: For human pancreas specimens, use 12.5 µg/ml mouse anti-human CD44v3 mAb or 12.5 µg/ml mouse IgG<sub>2bK</sub> as isotype control
- 10 Wash off primary antibody with PBS and transfer slides to slide container with 250 ml PBS. Wash 2 × 2min.
- 11 Wipe around sections using tissue and incubate with 26-54 µg/ml secondary HRP-rabbit anti-rat Ig (Dako) or alternative 30-60 µg/ml HRP-rabbit anti-rat IgG (Sigma). For use with primary CD44v3 mAb, incubate with 8 µg/ml secondary HRP-rabbit anti-mouse IgG (Invitrogen).

Note: for human pancreas sections, use 6.4 µg/ml secondary HRP-rabbit anti-mouse IgG (invitrogen).

- 12 Wash off secondary antibody with PBS and transfer to slide container with 250 ml PBS. Wash slides 2 × 2min.
- 13 Prepare AEC working solution: 4.75 ml acetate buffer (see Guidelines), 0.25ml AEC stock solution and 25 µl 3% H<sub>2</sub>O<sub>2</sub>. Filter using a disposable 0.2 µm filter. Use within 2 hours of preparation, refrigerate for short-term storage. Protect from light.
- 14 Wipe around sections using tissue and cover the sections with AEC solution for 30 min at room temperature.
- 15 Wash off AEC solution with deionized water and transfer slides to slide container with 250 ml deionized water. Wash 3x in 10 min.
- 16 Lightly counterstain with Gill's hematoxylin, wash in deionized water (2 x) and briefly dip in ammonium water (100 µl ammonia in 250 ml deionized water), 2 × 2 sec. Wash in deionized water (2x in 250 ml) and coverslip using glycergel mounting medium.
- 17 Image sections using a light microscope with camera attachment. Use Image J software with color deconvolution plugin to determine % of islet area stained.